

COMPARISON OF β -GALACTOSIDASES FROM NORMAL ($i^-o^+z^+$) AND
OPERATOR CONSTITUTIVE ($i^-o^cz^+$) STRAINS OF *E. COLI*

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Three genetic regions have been identified within the lactose (*Lac*) locus of *Escherichia coli* which specify the structure of the enzymes β -galactosidase, galactosidase permease, and thiogalactoside transacetylase.¹ The expression of the three loci has been shown to be regulated by the product of another genetic region (designated *i*) which is presumed to function by coordinately repressing the synthesis of these enzymes. The postulation of a repressor substance specified by the *i* locus leads to the prediction of a site of action for such a repressor. This site has been termed the "operator." The isolation of certain mutants which conform to the properties expected for such a controlling element supports the concept of a specific operator region.

Originally, Jacob and Monod characterized two different classes of mutants which defined the operator region. The first type was isolated from homozygous inducible diploids (i^+/i^+). These mutants had the properties of being constitutive even in the presence of a competent repressor (i^+). Thus, such constitutive organisms (termed o^c) were dominant over the i^+ genotype. Such dominance is in contrast to the usual constitutive state (i^-) which is recessive in the i^+/i^- diploid. Strains possessing the second class of mutants (o^o) used to define the operator region are unable to synthesize any of the three enzymes specified by the *Lac* region. However, these mutants mapped within a region contiguous with the extreme end of the β -galactosidase-determining structural gene (*z*). Recent studies by Beckwith have shown that the o^o cluster is not part of the operator locus and that these mutants should be defined as members of the class of polar mutations.² The o^c mutants remain as the only group which define the operator.

Jacob, Ullmann, and Monod³ have recently utilized the techniques of Beckwith to isolate a new series of operator constitutive mutants believed to represent deletions of varying sizes in the operator region (see *Materials and Methods*). They have examined the crude extracts from certain of these mutants in an effort to determine whether or not the galactosidase activities have properties similar to those of the wild-type protein. In all cases, the galactosidases, examined in crude extracts, were shown to have similar pH and temperature sensitivities. This paper reports the results of a continuation of these studies involving enzymic, physical, and chemical comparisons of the β -galactosidases isolated from an operator constitutive organism ($i^-o^cz^+$) with the "wild-type" enzyme from a regulator constitutive organism ($i^-o^+z^+$).

Materials and Methods.—Selection of operator constitutive strain: Two considerations were involved in the selection of the operator constitutive strain used here. First, it was important that the operator constitutive mutant used represent as large a deletion of the operator gene as possible. Second, the deletion mutation should include one of the terminal sections of the operator region. With regard to the first consideration Jacob *et al.*³ concluded that all operator constitutive mutations represented deletions of varying sizes. Based on this conclusion it was further inferred

that the degree of constitutivity is proportional to the length of the deletion, that is, the greater the deletion of the operator function the greater the constitutivity.⁴ To satisfy both criteria a strain was chosen in which both the operator function and the repressor function were completely deleted (Fig. 1). The mutant selected, designated o_{67}^c , has been shown to be a complete constitutive (i.e., absolutely no increased synthesis of β -galactosidase in the presence of inducer) even in the diploid $i^+o^+z^-/Fi^-o_{67}^cz^+$. The mutant was obtained through the generosity of Drs. F. Jacob and J. Monod.

Purification: The β -galactosidases employed in this study were isolated from the regulator constitutive strain of *Escherichia coli* K₁₂, 3300, and the operator constitutive strain K₁₂, 3000- o_{67}^c .

The growth conditions for each strain were identical and are described in detail elsewhere.⁶ The procedure for the purification of each enzyme, also described in detail in a previous publication, was briefly as follows. The lysate from approximately 1 kg of cells (wet weight) was fractionated with ammonium sulfate and the 0–40% fraction was passed through Sephadex G-200. The enzymically active fraction from G-200 was further purified by ion exchange chromatography using DEAE Sephadex A-50. The appropriate fraction from DEAE chromatography, homogeneous by polyacrylamide gel electrophoresis, was pooled and either stored under ammonium sulfate at 4° or carboxymethylated and stored in the inactive form.

Carboxymethylation: The protein, dissolved in an 8 M solution of recrystallized urea, was incubated at 38° with β -mercaptoethanol (0.1 M) for 4 hr and subsequently alkylated for 20 min at room temperature with a 5-fold molar excess of sodium iodoacetate prepared from recrystallized iodoacetic acid.⁵ The pH during the alkylation reaction was maintained at 8.2 by the addition of 6 N NaOH. The reduced-carboxymethylated product was dialyzed against 0.2 M NH_4HCO_3 , pH 8.2, and thoroughly lyophilized to remove the volatile salt.

Trypsin digestion: The reduced-carboxymethylated product was dissolved at a concentration of 5–10 mg per ml and dialyzed against 0.2 M NH_4HCO_3 , pH 8.2. Diisopropylfluorophosphate-treated trypsin, 2 \times crystallized (Worthington Biochem. Corp.), was added in a protein to enzyme ratio of 50:1 and the digestion allowed to proceed for 4 hr at 38°. The hydrolysates were lyophilized.

Reaction with cyanogen bromide: The reduced-carboxymethylated protein was dissolved in 70% formic acid at a concentration of approximately 5 mg per ml. A 50-fold excess of cyanogen bromide (Eastman Organic Chemicals) was added, the flask tightly stoppered, and the reaction⁶ allowed to proceed at room temperature overnight (approximately 18 hr). The resulting mixture was then frozen and lyophilized.

Electrophoresis: Polyacrylamide gel electrophoresis was performed with 7.5% "standard gel" according to the manual supplied by the Canal Industrial Corporation (Bethesda, Md.). Free electrophoresis was carried out utilizing a Perkin-Elmer model 38 electrophoresis apparatus. Equal quantities of native enzyme and enzyme from o_{67}^c cells were mixed to give a final protein concentration of 14 mg per ml. The mixtures were dialyzed against buffers⁷ at pH 6.0, 7.5, and 9.0. Electrophoresis was carried out at 0° for periods of time ranging from 4 to 6 hr.

Ultracentrifugal studies: A Spinco model E analytical ultracentrifuge equipped with both the standard schlieren and Raleigh interference optical systems was employed for all runs. Velocity measurements were carried out as described by Schachman on both enzyme preparations and on 1:1 mixtures of the two preparations.

Molecular weights were determined utilizing the high-speed equilibrium technique of Yphantis.⁸ All runs employed 3-mm columns of solution at speeds in the range of 7447 rpm and 8227 rpm, utilizing the AnJ rotor and protein concentrations of 0.1–0.3 mg/ml. All plate measurements were carried out using a Bausch and Lomb comparator.

Amino acid analyses: The lyophilized, carboxymethylated protein was suspended in 5.7 HCl in thick-walled hydrolysis tubes. The tubes were evacuated, sealed, and heated at 110° for 20 hr. Following hydrolysis, the samples were taken to dryness and chromatographed on a Beckman model 120B amino acid analyzer according to the technique of Spackman, Moore, and Stein.⁹

End-group analysis: Both the native and carboxymethylated galactosidases prepared from

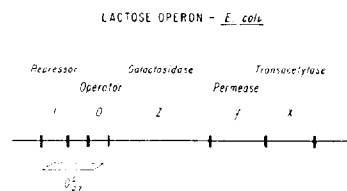


FIG. 1.—Schematic genetic map of the *Lac* operon showing the presumed deletion in strain o_{67}^c .

the 3300 and α_{67}^c strains were examined for NH_2 -terminal end groups by the fluorodinitrobenzene (FDNB) procedure.¹⁰ Reaction with FDNB was carried out in 0.1 *M* borate buffer containing 8 *M* urea at pH 9.0, 40°, with shaking for 4 hr. Urea was added to help ensure availability of susceptible groups to the reagent. Control experiments with oxidized ribonuclease, both in the presence and absence of 8 *M* urea, yielded 0.8 moles of NH_2 -terminal bis-DNP-lysine. The dinitrophenylated proteins were precipitated, washed, and hydrolyzed as described previously by Biserte.¹¹ Ether-soluble DNP-amino acids were separated and determined as described previously.¹² High-voltage electrophoresis¹¹ of the aqueous phase after ether extraction of the hydrolysate failed to reveal the presence of water-soluble DNP-amino acids.

Immunochemical comparison: Antisera were prepared as outlined by Balbinder and Preer¹³ using both the α_{67}^c and 3300 enzymes as antigens. Double diffusion utilizing agar plates was carried out according to the technique of Ouchterlony as described by Campbell *et al.*¹⁴ Quantitation of immunological activity was accomplished by the double-diffusion technique of Preer.¹⁵ The equivalence concentration of each enzyme was compared utilizing different antisera. Additional comparisons were carried out utilizing the "profile" technique of Oudin¹⁶ which has been modified by Finger and Heller.¹⁷ In this analysis, the two enzyme preparations are compared qualitatively and quantitatively by reacting them against several different antisera in Preer double-diffusion tubes.

Ultraviolet spectra: Ultraviolet spectral measurements were performed using a Cary model 15 recording spectrophotometer. Calculations for tyrosine to tryptophan ratios were done on spectral data obtained on carboxymethylated β -galactosidase dissolved in 0.1 *N* NaOH according to Beaven and Holiday.¹⁸

Renaturation: The kinetics of return of activity following inactivation in 8.0 *M* urea were followed after a 1:10 dilution of the urea-protein solution (1.0 mg/ml protein, 8 *M* urea, Tris 0.05 *M*, NaCl 0.1 *M*, mercaptoethanol 0.1 *M*, pH 7.5) into buffer lacking urea at room temperature. Aliquots were taken at various intervals for assay.

Results.—Behavior during purification: The course of the purification procedure, summarized in the *Materials and Methods* section, was identical in all respects for both enzymes. Purification of the two enzymes was conducted simultaneously, and the homogeneity of each product was established independently. The final products appeared to be homogeneous by polyacrylamide gel electrophoresis and yielded single boundaries in the analytical ultracentrifuge and during free electrophoresis. Free electrophoresis was carried out on 1:1 mixtures of the two enzyme preparations over a pH range of 6 through 9 (e.g., Fig. 2). Polyacrylamide gel electrophoresis patterns for the crude extracts, as well as for the final purified products (Fig. 3), were identical for both enzymes. The specific activities (340,000 units per mg) of the 3300 and α_{67}^c final products were equal within the precision of the measurements.

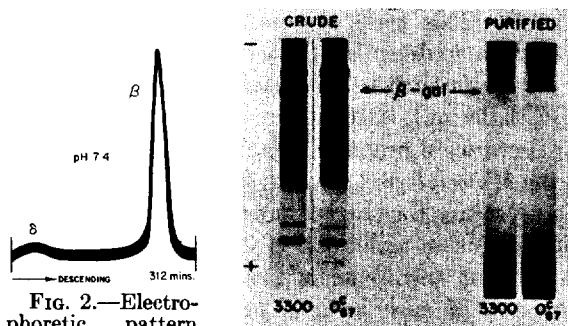


FIG. 2.—Electrophoretic pattern obtained at pH 7.4 of 1:1 mixture of β -galactosidases from strains α_{67}^c and 3300.

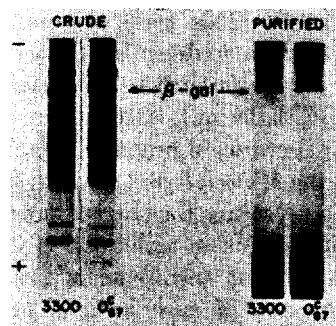


FIG. 3.—Polyacrylamide gel electrophoresis patterns of crude extracts and purified β -galactosidases of strains α_{67}^c and 3300.

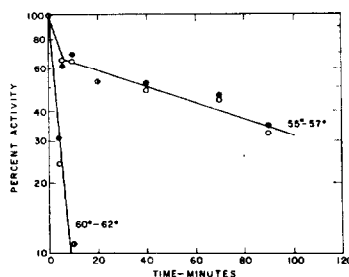


FIG. 4.—Kinetics of inactivation of the two purified galactosidases at 55–57° and 60–62°. ●, β -galactosidase from strain 3300; ○, β -galactosidase from strain α_{67}^c .

Furthermore, no significant differences in K_m , determined using the Lineweaver-Burk plot were observed between the two enzyme preparations.

Sensitivity to heat and urea: The kinetics of inactivation of the two enzyme preparations were examined at three different temperatures. No significant difference was observed (Fig. 4) between the kinetics of inactivation of the two enzyme preparations at 55–57° and 60–62°C.

Renaturation from 8 M urea: Zipser has shown that β -galactosidase can be renatured to a large extent from 8 M urea upon removal of the urea by simple dialysis.¹⁹ The kinetics of this renaturation process can be conveniently studied utilizing direct dilution of the 8 M urea solution into buffer at room temperature containing Mg^{++} , Na^+ , and mercaptoethanol (buffer B⁵). These studies have shown that the renaturation from 8 M urea represents a refolding of the extended chain (mol wt = 135,000) and subsequent aggregation into the native enzyme, having a molecular weight of 540,000. It would appear, therefore, that a study of the kinetics of renaturation from 8 M urea might be a sensitive measure for comparison of the complex forces involved in the determination of the tertiary and quaternary structure of the enzyme. In support of this, it should be pointed out that the heat-labile enzyme produced by a reversion mutant of the polar mutation, o^{84} , (o^{84} rev-1) is completely unable to renature from 8 M urea under conditions which result

in the successful renaturation of the galactosidase of strain 3300.

Figure 5 shows the kinetics of reactivation when the two enzymes are diluted from 8 M urea. This test reveals no difference between the two enzyme preparations.

Molecular weights: The molecular weight of native β -galactosidase reported by Sund and Weber²⁰ has been corroborated by Craven *et al.*⁵ Using the high-speed equilibrium technique of Yphantis,⁸ the molecular weights of the o_{67}^c and 3300 preparations were found to be 550,000 and 530,000, respectively. These values are in marked contrast to that determined for the enzyme produced by the CRM mutant, 3310, which was found to have a molecular weight of 235,000.²¹ This type of comparison is, of course, only of value in detecting gross differences in quaternary structure such as that produced in the case of the CRM mutant, 3310.

Immunochemical comparison: Antisera were prepared against the purified 3300 and o_{67}^c preparations as described in the *Materials and Methods* section. In both the homologous and heterologous reaction systems, the two enzyme preparations showed complete identity utilizing the double-diffusion technique of Ouchterlony (Fig. 6). For quantitative tests, the double-diffusion technique of Preer was employed to determine the equivalence concentration of each antigen preparation against a standard antiserum prepared against β -galactosidase of strain 3300 ($i-o^+z^+$). In such tests, precise quantitative results may be obtained for any antiserum (or antigen) which shows some degree of cross reactivity with an antigen (or anti-

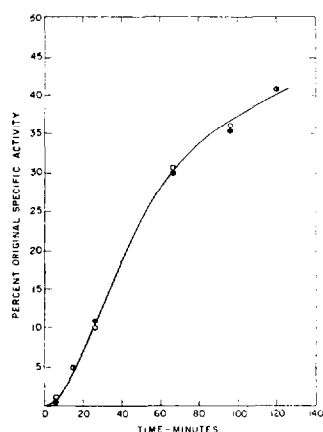


FIG. 5.—Kinetics of reactivation of β -galactosidases from strains o_{67}^c and 3300 following dilution from 8 M urea solutions.

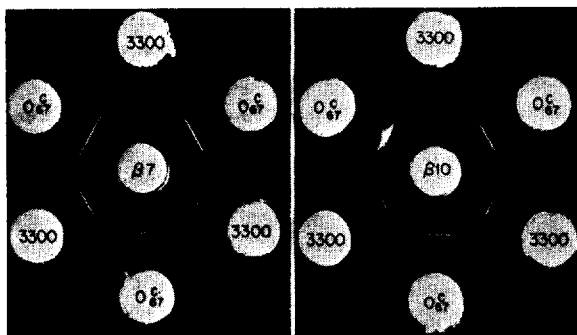


FIG. 6.—Ouchterlony double-diffusion patterns obtained with β -galactosidases from strains o_{67}^c and 3300 using as antisera in the center wells, anti- o_{67}^c ($\beta 7$) and anti-3300 ($\beta 10$).

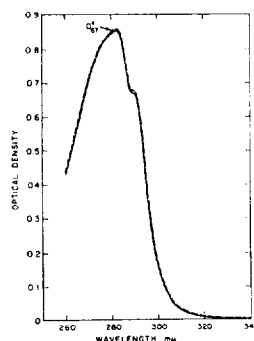


FIG. 7.—Ultraviolet spectra of β -galactosidases from strains o_{67}^c and 3300. o_{67}^c , ----; 3300, —.

TABLE 1

QUANTITATIVE PRECIPITIN ANALYSIS* OF β -GALACTOSIDASES OF STRAINS 3300 AND o_{67}^c

Antigen	$\mu\text{g./ml Serum}$	Average
3300 ($i^-o^+z^+$)	1220	1290
	1350	
	1310	
	1280	
$o_{67}^c(i^-o^+z^+)$	1360	1350
	1400	

* Reactions were performed at equivalence. Values reported are μg antigen precipitated/ml antiserum (prepared against enzyme from strain 3300).

serum). Table 1 presents the results with two different antisera, comparing the two antigens, o_{67}^c and 3300. No significant antigenic differences were observed between these proteins in their ability to form precipitates with apparently homologous antibodies.

A third immunological procedure (termed "profile analysis") which can yield a critical comparison of proteins is the technique of Oudin,¹⁶ which has been usefully modified by Finger and Heller.¹⁷ The latter modification utilizes the Preer double diffusion technique for comparative analysis. It is possible, using the profile technique, to compare the reactions of a given antigen with a large number of antisera. The position of the precipitin band in the Preer tube reflects the level of "recognition" of the antigen-antibody reaction. Each antigen will thus give a characteristic "profile" against a series of antisera to which any other antigen preparation can be compared. The purified preparations of β -galactosidase from the 3300 and o_{67}^c strains were tested by this method against five antisera to the 3300 protein. These sera had been prepared independently using five different rabbits. The relative reactivities of the two antigens to the five antibody preparations were constant, within the precision of the measurements, against all five sera. It is apparent that, by immunological methods, no differences are evident between the enzymes studied here.

Ultraviolet spectra: The absorption characteristics of the two enzymes, o_{67}^c and 3300, were examined by scanning the two preparations after adjustment to approximately equal concentrations. Figure 7 is a direct tracing of such an analysis where consecutive scans were superimposed upon each other. An analysis of these curves shows no differences and indicates identical maximum extinctions at 282 $m\mu$, and 280/260 $m\mu$ ratios. Furthermore, there is no apparent difference between the ratios of tyrosine to tryptophan in the two proteins as shown by the spectra of the carboxymethylated proteins in 0.1 *N* NaOH.

Amino acid analysis and peptide mapping: The amino acid compositions of the two enzymes were determined after acid hydrolysis using a Beckman-Spinco automatic amino acid analyzer. The composition of the protein from the 3300 strain has been reported previously⁵ and the analyses of the β -galactosidase from o_{67}^c yielded identical results within the error of the method (approx. 3%).

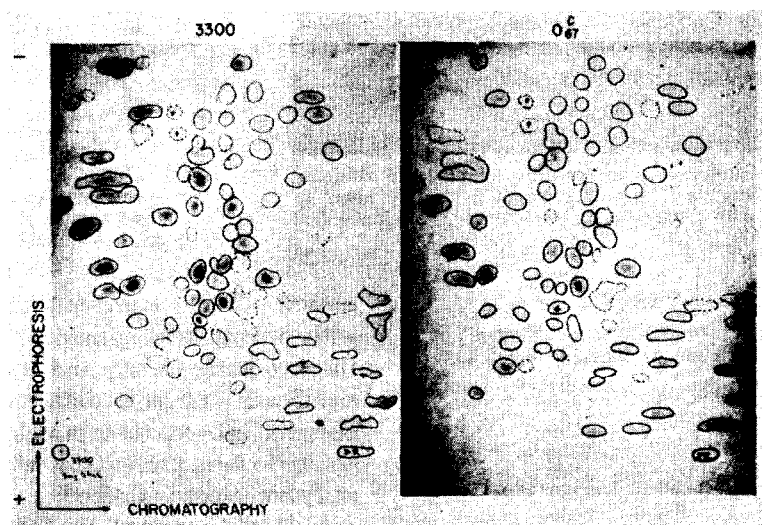


FIG. 8.—Peptide maps obtained from trypsin digests of the galactosidases isolated from the constitutive strain (3300) and an operator constitutive mutant strain (o_{67}^c). Phenol red (PR) was added as a marker in the chromatographic dimension.



FIG. 9.—Polyacrylamide gel patterns of CNBr digests of carboxymethylated β -galactosidases of strains o_{67}^c and 3300. Pattern A, 5% cross-linked gel; pattern B, 7½% cross-linked gel.

A more direct chemical comparison is afforded by the analysis of the peptide patterns obtained on tryptic digests of the proteins.²² Figure 8 shows the patterns which were obtained using digests of the carboxymethylated enzymes from strains 3300 and o_{67}^c . The minor differences observed were also seen in duplicate maps of a single protein preparation.

CNBr fragmentation: A second direct comparison of primary structures was possible by analysis of the products produced by the specific action of cyanogen bromide. Cyanogen bromide has been demonstrated to cleave, specifically, peptide linkages following methionine residues.⁶ This technique has been used extensively with the "standard" protein of strain 3300.²³ Figure 9 presents the results of such cleavage of both the reduced alkylated β -galactosidase chains of strains 3300 and o_{67}^c . Two different cross-linked polyacrylamide gels were utilized to compare the CNBr fragments

of the two proteins. The patterns have shown no reproducible differences. Should a significant deletion of information exist in the o^c strain, such a genetic property might be expected to result in a significant alteration (or disappearance) of at least one of the resulting polypeptide fragments.

End-group analysis: A deletion of the genetic region specifying the NH_2 -terminal structure of β -galactosidase would increase the probability of detection of a new NH_2 -terminal end group. Applications of the FDNB procedure resulted in the recovery, from both the o_{67}^c and 3300 strains, of approximately 0.6 moles of threonine per 135,000 mol wt.

Discussion.—As part of their original generalized concept of the *Lac* operon, Jacob and Monod¹ postulated three functions for the operator locus. It was suggested that the operator site was responsible not only for the “recognition” of the repressor substance and the initiation of message translation, but also for the specification of the proximal portion of the β -galactosidase protein itself. This latter function was deduced from the properties of a series of mutations of the polar variety, termed o^0 mutations, which mapped approximately in the same area as the operator constitutive mutations. Since several reversions of o^0 mutants produced temperature sensitive β -galactosidase, it was inferred that the operator region specified the terminal structure of the enzyme itself. However, the experiments by Beckwith² demonstrated that the o^0 mutations are not within the operator locus but represent polar mutations of the β -galactosidase structural cistron. Thus, there remained no direct evidence that the operator specifies any portion of the β -galactosidase molecule.

The present studies, involving chemical, physical, and immunological examination of the β -galactosidases produced by the wild-type and the operator constitutive mutant, o_{67}^c , are consistent with the complete identity of these proteins. Perhaps most significant are the chemical results which include identical peptide maps, polyacrylamide gel patterns of cyanogen bromide fragments, and end-group data. The latter determinations involve some uncertainty because of the difficulty of detecting such NH_2 -terminal residues as proline, glycine, histidine, and the acetyl-amino acids in general. Nevertheless, the same terminal residues were observed for both the normal and operator constitutive materials, and no *new* groups were detected in the latter protein.²⁴ Since the deletion in o_{67}^c is believed to extend from within the operator through the *i* locus (see *Materials and Methods*), the terminal region of the operator must be deleted. If this characterization of the o_{67}^c mutation is correct, the present end-group data suggests that the proximal portion of the *Z* gene has not been altered and that the operator is not part of this structural gene.

Summary.—The properties of purified β -galactosidases from a normal constitutive strain of *E. coli* K12 (3300, $i^-o^+z^+$) and from an operator constitutive mutant (o_{67}^c , $i^-o^c z^+$) have been compared using physical, chemical, and biological methods. No detectable differences were found between the two protein preparations. It is concluded that the operator locus does not specify any part of the structure of the β -galactosidase molecule.

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